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PRINCIPAL INVESTIGATOR: Stephen D. Hursting, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
MD Anderson Cancer Center
Houston, Texas 77030

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Stephen D. Hursting, Ph.D.

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MD Anderson Cancer Center
Houston, Texas 77030

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The ectopic expression of the *Wnt-1* proto-oncogene in *Wnt-1* transgenic (*Wnt-1 TG*) mice results in mammary gland hyperplasia and early adenocarcinoma development. Mammary tumorigenesis is further accelerated in p53-deficient *Wnt-1 TG* mice generated by mating *Wnt-1 TG* mice with p53-knockout mice. The purpose of the proposed study is to evaluate the effects of calorie restriction and the chemopreventive agents dehydroepiandrosterone, genistein and N-(4-hydroxyphenyl)retinamide on spontaneous mammary tumorigenesis in *Wnt-1 TG* mice with and without p53-deficiency. We are also evaluating the mechanisms underlying interventions that modulate spontaneous mammary tumorigenesis in these mice by measuring the expression of *Waf-1/p21* (a p53-related cell cycle regulator), *Bcl-2* (a p53-related apoptotic regulator), retinoic acid receptor β (associated with the chemopreventive efficacy of N-(4-hydroxy-phenyl)retinamide and *Brca-1* (a putative tumor suppressor gene associated with familial breast cancer). This study will provide the initial characterization of *Wnt-1 TG* mice and p53-deficient *Wnt-1 TG* mice as models of spontaneous mammary tumorigenesis for cancer prevention studies. We have successfully developed a colony of these mice over the course of the year and have generated mice for the initial aim of the study, which is to assses the effects of our interventions on spontanoeus mammary tumor development in these mice.

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Stephen P. Hunter 9/28/97

PI - Signature Date

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Characterization of Wnt-1 Transgenic Mice (With and Without p53-Deficiency) as Models of Spontaneous Mammary Tumorigenesis for Chemoprevention Studies

Principal Investigator: Stephen D. Hursting, Ph.D., M.P.H.

Institution: M.D. Anderson Cancer Center
1515 Holcombe Boulevard
Houston, TX 77030

Phone: (713) 745-2492

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1.0 INTRODUCTION

1.1 Wnt-1 Transgenic Mice

Future progress in the chemoprevention of breast cancer may be facilitated by the use of animals with specific genetic susceptibility for spontaneous mammary tumorigenesis. This may overcome some of the limitations of existing carcinogen models, which are characterized by high dose, acute exposures of a gentoxic carcinogen generally not encountered by humans. *Wnt-1* transgenic (*Wnt-1 TG*) mice are predisposed to the development of mammary adenocarcinomas. *Wnt-1* is a mouse proto-oncogene which encodes a cysteine-rich glycosylated secretory protein normally expressed during mouse embryonic development and not expressed in the normal mammary gland (1). Both male and female *Wnt-1 TG* mice demonstrate high-level mammary gland expression of *Wnt-1* mRNA as well as marked proliferation of alveolar and ductal mammary epithelium. All females develop mammary carcinomas by 41 weeks of age (median time to development of palpable tumor is 23 weeks of age) whereas only 15% of males develop mammary tumors by 1 year of age, suggesting other factors, such as hormones, interact with *Wnt-1* overexpression to lead to mammary tumor formation in these transgenic animals (2).

Collaborator L. Donehower and colleagues have reported that the kinetics and histology of mammary tumor formation resulting from

the ectopic expression of *Wnt-1* are greatly influenced by the presence or absence of the p53 tumor suppressor gene. The median age of onset of mammary tumors in the female offspring of *Wnt-1 TG* mice crossed with $p53^{-/-}$ mice is approximately 12 weeks (compared to 23 weeks in *Wnt-1 TG* mice with wild type p53), and all of the p53-deficient *Wnt-1 TG* mice form mammary tumors by 15 weeks of age (compared to 41 weeks in *Wnt-1 TG* mice with wild-type p53). In addition, mammary tumors from *Wnt-1 TG* mice with wild-type p53 tend to be more organized, fibrotic, and differentiated relative to tumors from p53-deficient *Wnt-1 TG* mice (3). **The responsiveness of either the *Wnt-1 TG* mice or the p53-deficient *Wnt-1 TG* mice to chemopreventive agents or other tumor modulating regimens has not previously been studied.**

1.2 Chemoprevention Studies in p53-Deficient Mice

We have previously shown that several nutritional and chemopreventive interventions delay the onset of spontaneous tumorigenesis in male and female $p53^{-/-}$ mice. Since the proposal was submitted, we have reported that calorie restriction (CR), a well-documented and potent modulator of rodent tumor development, including carcinogen-induced mammary tumors, significantly delayed spontaneous tumorigenesis and slowed lymphocyte cell cycle traverse in male $p53^{-/-}$ and wild-type ($p53^{+/+}$) mice (4, 5). The tumor-delaying effect of CR was virtually identical in the two genotypes, even though the rapidity with which tumors developed was much faster in $p53^{-/-}$ than $p53^{+/+}$ mice, indicating that the mechanism underlying the anti-tumor effect of CR is p53-independent.

We also reported (6) that dehydroepiandrosterone (DHEA), a steroid with anti-inflammatory and cancer chemopreventive activity in several tumor models, including chemically induced mammary tumor models (7), delayed spontaneous tumorigenesis by over 80% and specifically suppressed lymphoma development in male $p53^{-/-}$ mice. Also, the DHEA analogue 16-a-fluoro-5-androsten-17-one, which may be a more promising chemopreventive agent given its lack of androgenic and estrogenic activity and appetite suppression relative to DHEA, also suppresses spontanous lymphoma development (8). In addition, we found that DHEA and its analogue increased thymic expression of the p53-dependent cell cycle regulator *Waf-1/p21* and

decreased thymic expression of the p53-related apoptotic regulator *Bcl-2* (with no effect on *Bax*, a negative inhibitor of *Bcl-2*) in both p53-knockout and wild-type mice (9). We also found that these chemopreventive steroids in our hands do not decrease nucleotide pools (their purported mode of action; 8) but do decrease nitric oxide generation and down regulate the expression of the inducible nitric oxide synthetase gene (10).

We have not yet evaluated the effect of 4-HPR on spontaneous tumorigenesis in $p53^{-/-}$ mice. However, preliminary data in male $p53^{-/-}$ mice in our laboratory suggest that 4-HPR increases thymic expression of *Waf-1/p21* and decreases the expression of *Bcl-2* but has no effect on *Bax* expression. These changes in gene expression are consistent with those observed in response to DHEA and other efficacious interventions in our laboratory (9,11). 4-HPR has been reported to increase expression of retinoic acid receptor (RAR)-β in normal mammary epithelial cells, but not tumor cells (12). Collaborator R. Lotan and colleagues have shown increased RAR-β to be associated with the chemopreventive efficacy of retinoids in head and neck cancers (13) and also in the response of nitrosomethylurea-induced mammary tumors in Fischer rats to 4-HPR (personal communication). We also found that the soy isoflavone genistein exerted a modest delay in spontaneous tumorigenesis in male, but not female, p53-/- mice (14).

2.0 Purpose and Scope of the Work

The purpose of the ongoing study is to evaluate the effect of calorie restriction (CR; the most potent and broad acting dietary perturbation for inhibiting rodent tumor development) and the chemopreventive agents DHEA, genistein and 4-HPR, on spontaneous mammary tumorigenesis and relevant gene expression in *Wnt-1 TG* mice with and without p53-deficiency. This will provide the initial step in characterizing these mice as models of spontaneous mammary tumorigenesis for cancer chemoprevention studies.

The specific aims of the proposed study are:

1. To determine whether CR, DHEA, genistein or 4-HPR modulate the incidence, latency (time to palpable tumor) or burden (total number of mammary tumors and total tumor weight) of spontaneous mammary tumors in *Wnt-1 TG* mice with or without wild-type p53 expression.

2. To characterize the mechanisms underlying interventions that inhibit mammary tumorigenesis by measuring in non-neoplastic mammary tissue collected 2 weeks after the onset of treatment (before tumor development) the expression (relative to controls) of the p53-dependent cell cycle regulator *Waf-1/p21* by northern blot analysis as well as the p53-related apoptotic regulator *Bcl-2* and its negative inhibitor *Bax* by a competitive polymerase chain reaction (PCR) assay.

3. To evaluate the expression of RAR-β by in situ hybridization in mammary tumors (collected throughout the study) as well as in non-neoplastic mammary tissue collected after two weeks of treatment.

4. To evaluate the expression of *Brca-1* in mammary tumors (collected from the tumor study) and in non-neoplastic mammary tissue after 2 weeks of each treatment.

2.0 BODY

2.1 Original Statement of Work

Technical Objective 1: Treatment Effects on Spontaneous Mammary Tumorigenesis in *Wnt-1 TG* and p53-Deficient *Wnt-1 TG* Mice.

Task 1: Months 1-4. Mate wild-type females x *Wnt-1 TG* male mice and mate $p53^{-/-}$ females by p53-deficient *Wnt-1 TG* males to produce 80 female wild-type, 80 female *Wnt-1 TG* and 80 female p53-deficient *Wnt-1 TG* mice for the study.

Task 2: Months 2-4. Genotype female offspring of above matings; randomize relevant genotypes into treatment arms.

Task 3: Months 2-15. Continue mice on diet treatments; monitor food consumption, body weights and mammary tumor development; kill mice when tumors are 1.5-2 cm in diameter; collect and store portions of mammary and other tissues in formalin for subsequent histopathologic analysis or flash freeze in liquid nitrogen and store at -80^0 C for subsequent molecular analyses.

Task 4: Months 16-19. Conduct histopathologic analysis.

Technical Objective 2: Treatment Effects on *Waf-1/p21*, *Bcl-2*, and *Bax* Expression in Non-Neoplastic Mammary Glands from *Wnt-1 TG* and p53-Deficient *Wnt-1 TG* Mice.

Task 5: Months 16. Randomize an additional 15 mice per genotype (3 mice/treatment group) to each of the 5 dietary treatments.

Task 6: Month 17. Collect mammary glands and other tissues after 2 weeks of treatment; store half in buffered formalin; flash freeze the remainder and store at -80^0 C

Task 7: Month 18. Isolate total RNA from frozen mammary tissues.

Task 8: Months 19-24. Analyze *Waf-1/p21* expression by Northern blotting and *Bcl-2* and *Bax* expression by competitive PCR analysis.

Technical Objective 3: Treatment Effects on RAR-β Expression in Mammary Tumors and Non-Neoplastic Mammary Glands.

Task 9: Months 2-15. Collection and storage of mammary tumors in buffered formalin.

Task 10: Month 18. Preparation of tissue sections for in situ hybridization.

Task 11: Months 19-24. Analysis of RAR-β expression by in situ hybridization.

Technical Objective 4: Treatment Effects on *Brca-1* Expression.

Task 12: Month 18. Isolate total RNA from frozen non-neoplastic mammary glands (collected and isolated as described in Tasks 6 and 7) and from frozen mammary tumors (collected as described in Task 3).

Task 13: Months 19-24. Analyze *Brca-1* expression by northern blot analysis.

2.2 Progress report

2.2.1 Generation of Wild-Type, *Wnt-1 TG*, and p53-Deficient *Wnt-1 TG* Mice.

Our initial step was to develop an animal colony at the M.D. Anderson Cancer Center to generate the needed mice for the study. Our funding began 10/1/96, and we had obtained 4 breeding pairs on 7/26/96 from Dr. Larry Donehower (Baylor College of Medicine), consisting of p53-/-:Wnt-1 TG males x Wnt-1 TG females, to begin the colony.

The development of this colony presented several challenges which we have solved through our breeding strategies. First, the p53-/- females have a high rate of dystochia, and thus experience difficulty with labor. There is also a significant gender skew in the p53-/- mice, with approximately a 4:1 ratio of males to females. In addition,

the Wnt-1 TG female does not lactate. We have thus established a colony consisting of 50 male p53+/-:Wnt-1 TG mice and 25 female p53+/- mice, and have generated 402 mice thus far to establish the colony and begin to seed the tumorigenesis study. In an effort to enhance our numbers of female p53-/-:Wnt-1 TG mice, we also set-up (month 9) 10 breeding pairs of p53+/- males x p53+/-:Wnt-1 TG females, and use 10 C3H mice to foster the pups. Our original estimates for colony development did not anticipate these challenges; thus our estimate of only 4 months to generate the mice underestimated the amount of time required to develop the breeding colony and accomplish this task. All mice had been required to establish the colony until Month 10, when we had sufficient numbers to begin the tumor study.

2.2.2 Genotyping procedure for Wnt- and p53

Southern blotting had previously been used to genotype Wnt-1 TG and p53-/-:Wnt-1 TG mice, but we required a faster and less time-intensive method for the genotyping of the large numbers of mice we are generating. We therefore developed a PCR-based genotyping procedure for Wnt-1 which is used in tandem with our PCR-based protocol for p53 that we routinely use with our p53 colony. The protocol is as follows:

PCR PROTOCOL
(allelotype determination for WNT-1 transgenic mice)

Presence of a 350 bp product indicates the WNT-1 transgene is present. Absence of this band indicates the WNT-1 transgene is not present. A separate PCR (using different primers) should be performed on each sample to verify the integrity of the template/PCR process. Control DNAs supplied include W666 (p53 -/-; wnt-1 +), W672 (p53 +/-; wnt-1 -), W708 (p53 +/+; wnt-1 +), and W663 (p53 +/+; wnt-1 -).

PCR Primer Sets		**Product size**
WNT-1 (sense)	5'-GGACTTGCTTCTCTTCTCATAGCC-3'	
SV40 (antisense)	5'-CCACACAGGCATAGAGTGTCTGC-3'	350 bp

This PCR product runs closer to 400 bp rather than 350 bp

The SV40 primer is the "L" (late) or "plus" strand of SV40 at position 4198-4220. It serves as the "antisense" primer in this reaction because the transgene vector was constructed in this fashion.

Amplification Reagents

Rxn Buffer (10X)	2.5 ul
dNTP mixture @ 2.5 mM each	1.0 ul
5' primer (15 pmol)	

3' primer (15 pmol)	
Taq Polymerase (5U/ul, 1.5 U)	0.30 ul
H_2O to 25.0 ul	
DNA (Positive Control, ie. heterozyg. DNA)	>200.0 ng
DNA (Sample, ie. proteinase K prep)	1.0 ul
No DNA (ie. H_2O)	5.0 ul
Mineral Oil	2 drops

Amplification Cycle Conditions (Perkin Elmer PCR Kit)

Preheat thermocycler to 94°C

File 512- 3194°C 2 min 1 Cycle

File 512- 3294°C 30 sec
60°C 30 sec
72°C 1 min 40 Cycles

File 512- 10Soak 4°C

Add 5 ul of loading buffer (5X) to each reaction. Load 15ul (or more) per well on a 2% agarose gel.

2.2.3 Spontaneous Mammary Tumor Study

A randomized block design is being used, with the goal of 20 female wild-type mice, 80 female *Wnt-1 TG* mice and 80 p53-/-: Wnt-1 TG mice. The Wnt-1 TG and p53-/-:Wnt-TG mice will be randomized to one of five treatment groups (total of 16 mice/group): (1) control (AIN-76A diet only); (2) CR (60% of control carbohydrate calorie consumption and 100% of all other nutrients); (3) DHEA (AIN-76A diet containing 0.3% w/w DHEA); (4) genistein (AIN-76A diet containing 0.04% w/w genistein); 5) 4-HPR (AIN-76A diet containing 8 ppm 4-HPR). The wild-type mice are being administered control diet and will serve as a comparison group; since the original submission, we have completed spontaneous tumor studies with female p53+/+ and p53+/- mice and have not observed a single mammary tumor in these mice.

For the tumor study, we have thus far completed the generation of wild-type females (n=20) and have generated 14 Wnt-1 TG females. These mice were started on study on September 1, and are currently under treatment, with the 20 wild-types receiving control diet and 14 Wnt-1 TG females receiving control (n=7) or CR (n=7). We have also generated 5 p53-/-:Wnt-1 TG females (3 on control diets and 2

on CR). All mice are 5 weeks old when randomized into the treatment groups. The generation of sufficient numbers of female p53-/-:Wnt-1 TG mice has presented the greatest challenge, although the addition of the fostering group should dramatically increase our numbers, as our genotyoing results from the most recent matings indicate approximately 25 p5-/-:Wnt-1 TG mice from that mating alone. Thus, by October 8, we anticpate a total of 30 p53-/-:wnt-1 TG mice, along with a total of 51 Wnt-1 Tg mice, based on the recent genotyoping results. Thus, we should complete all the groups by the end of this year. Also, we are randomizing male p53-/-:Wnt-1 TG mice into the same treatments as the females at no cost to the study. The male p53-/-:Wnt-1 TG also demonstrate tremendous susceptibility to spontaneous mammary tumorigenesis, with nearly 100% of thos emice developing mammary tumors by 25 weeks of age. We have thus far randomized 4 of these mice into the study (2 controls, 2 CR).

Food is being administered *ad libitum* to all groups with the exception of the CR group, which receive daily aliquots of food based on 60% of the control group's food consumption. All mice are individually housed and begin tratments by 35 days of age. All food is purchased fromResearch Diets, Inc., (New Brunswick, NJ). Food consumption and body weights are measured weekly, and each animal is palpated twice weekly to determine date of appearance of detectable mammary tumor (~0.5 cm in diameter, as measured by caliper). When a tumor reaches 1.5 cm in diameter, the tumor-bearing mouse will be killed; individual tumors excised, counted and weighed; and half of the tumor and non-tumor mammary gland tissue sections will be stored in buffered formalin for histopathologic analysis, to be performed by the Veterinary Services Core Facility at the M.D. Anderson Science Park-Research Division. The remaining mammary tissue will be flash frozen in liquid nitrogen for molecular analyses. Any mice alive 1 year after randomization will be killed and their mammary glands removed and analyzed as described for tumor-bearing mice.

Our oldest p53:Wnt-1 TG female is only 9 weeks old, so no tumor development in our mice has yet to be observed. We know from our breeding colony that the female p53:Wnt-1 TG mice remain tumor free for approximately 10-11 weeks, then tumors rapidly develop thereafter, with >1.5 cm tumors present within 1-2 weeks after the initial detection of a palpable tumor. To make up for some of the increased time needed to establish the colony, we have arranged

expedited histopathology processing with our pathology core. Also, rather than wait for the completion of our histopathologic analysis to begin our intermediate endpoint studies, we will proceed wit hthose studies based on the survival and gross pathogic findings from our tumor study. Thus, we should be able to maintain our original time-line to accomplish Technical Objectives 2, 3 and 4.

Conclusion

Due to the nature of this study, with much of the first year occupied by generating the mice for the spontaneous tumorigenesis study, we can only report our progress towards that goal. However, our results will come quickly now as we complete the study groups and begin to observe tumor development. We have made significant progress developing the animal colony and identifying breeding schemes which overcome the husbandry challenges inherent in these mice. We have also developed a PCR-based genotyping procedure to expedite that aspect of the experiment. With the development of the colony now complete, we have begun the spontaneous tumorigenesis study and anticipate the initial results on the response to calorie restriction from the p53-/-:Wnt-1 TG mice within the next few months, and with the Wnt-1 TG mice by early 1998.

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